AN ASSAY

Cross-Reference to Related Application.

This application claims priority from U. S. Provisional Application No. 60/179,948, filed on February 3, 2000.

FIELD OF THE INVENTION

The present invention relates generally to an assay for detecting variant Hepatitis B viruses (HBVs) which exhibit altered sensitivity to agents. The variant HBVs are delivered to cells using a baculovirus vector. The same agents generally have a particular effect or absence of effect on a reference HBV. The altered sensitivity is in relation to the effects of the agent on one or more stages of infection, replication, assembly or release of virus or virus-like particles including any intermediary steps during the processes of viral infection, replication, assembly and/or release. The identification of variant HBVs with altered sensitivities to anti-HBV agents provides a means of monitoring cross resistance, or the development of new therapeutics effective against variant HBVs with altered sensitivities to other anti-HBV agents, as well as monitoring therapeutic protocols which may then need to be modified to ensure the appropriate anti-HBV agent is administered or that the appropriate therapeutic protocol is instituted. The present invention further provides variant HBVs detected by the assay of the present invention and to components thereof as well as recombinant, chemical analogue, homologue and derivative forms of such components.

BACKGROUND OF THE INVENTION

Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in Australia or any other country.

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.

Specific mutations in amino acid sequence are represented herein as "Xaa_1nXaa_2" where Xaa_1 is the original amino acid residue before mutation, n is the residue number and Xaa_2

is the mutant amino acid. The abbreviation "Xaa" may be the three letter or single letter amino acid code. A mutation in single letter code is represented, for example, by X_1nX_2 where X_1 and X_2 are the same as Xaa_1 and Xaa_2, respectively. The amino acid residues for Hepatitis B virus DNA polymerase are numbered with the residue methionine in the motif "Tyr Met Asp Asp (YMDD)," being residue number 550.

The reference HBV is considered herein to comprise a composite or consensus nucleotide or amino acid sequence from HBV genotypes A through G (1, 2).

The rapidly increasing sophistication of recombinant DNA technology is greatly facilitating advances in the medical and allied health fields. This is particularly the case with the generation of therapeutic compositions and recombinant vaccines. Recombinant technology is providing the genetic bases for screening or identifying useful components for therapeutic compositions.

Hepatitis B virus (HBV) can cause debilitating disease conditions ranging from subclinical infection to chronic active hepatitis and can lead to acute liver failure or fulminant hepatitis.

Most patients will suffer an acute hepatitis during which time the virus is eliminated. In fulminant hepatitis, patients have acute liver failure and this frequently leads to patient death. About 5% of patients in North America and Europe fail to eliminate the virus, whereas in West Africa, up to 15% of infected patients fail to clear HBV (3). Persistent HBV infection predisposes the host to chronic liver disease and hepatocellular carcinoma (4).

The HBV genome comprises a series of overlapping genes in a circular, partially double-stranded DNA molecule (5) [see also Figure 1]. These genes encode for four overlapping open reading frames. For example, the gene encoding the DNA polymerase overlaps the viral envelope genes (Pre-S1, Pre-S2 and S) and partially overlaps the X and core genes. The protein component of the small HBV surface protein is generally referred to as the

HBV surfacc antigen (HBsAg) and is encoded by the S gene sequence. The Pre-S1 and Pre-S2 gene sequences encode the other envelope components (6). The core open reading frame encodes for both the hepatitis B core protein (HBcAg) and HBeAg, which starts from a precore initiation codon. HBV variants can have single or multiple mutations in one or more of the overlapping genes.

The HBV DNA polymerase is a reverse transcriptase (i.e. an RNA dependent DNA polymerase) and also has DNA dependent DNA polymerase as well as primase and RNase H activity. Nucleoside analogues have been used to inhibit HBV DNA replication. However, mutations have arisen in the gene encoding the HBV DNA polymerase resulting in the development of HBV variants resistant to the nucleoside analogues. Resistance may occur to a single nucleoside analogue or cross-resistance may also occur to an entire family of nucleoside analogues. Furthermore, when the mutation occurs in a region overlapping with the gene encoding HBsAg, alterations may occur to the HBsAg itself leading to the development of vaccine escape mutants.

Some precore variants of HBV result in hepatitis B e antigen (HBeAg)-negative hepatitis B. Seven to 30% of patients with chronic HBV infection worldwide are HBeAg-negative and are positive for HBV DNA by hybridisation using commercial tests. One such variant is unable to synthesize HBeAg. A single base substitution (G-to-A) at nucleotide 1896 (A_{1896}; numbering from the unique *Eco*RI site) gives rise to a translational stop codon in the second last codon (codon 28) of the precore gene. Other precore and basal core promoter (BCP) mutations are listed in Table 1. Since the core gene itself is not affected, synthesis of the core protein proceeds normally enabling production of virions. Precore A_{1896} mutations occur in both anti-HBe-positive patients with mild disease and those with high level viraemia and severe chronic hepatitis, suggesting that there is not a direct causal association with chronic progressive disease. However, infection with precore mutant virus has been associated with fulminant hepatitis and in the transplantation setting, graft failure (15).

The HBsAg comprises an antigenic region referred to as the "a" determinant (7). The "a" determinant is complex, conformational and dependent upon disulphide bonding among highly conserved cysteine residues. Genetic variation leading to changes in the "a" determinant has been implicated in mutants of HBV which escape the immunological response generated to conventional vaccines (8-12). One particularly common mutation is a glycine (G) to arginine (R) substitution at amino acid position 145 (G145R) of HBsAg. This mutation affects the "a" epitope region.

The increasing reliance on chemical and immunological intervention in treating or preventing HBV infection is resulting in greater selective pressure for the emergence of variants of HBV which are resistant to the interventionist therapy. Due to the overlapping genomic structure of HBV, HBV variants, may be directly or indirectly selected for by the use of chemical agents or vaccines.

It is important to be able to detect variant HBVs so that appropriate steps can be taken to modify a therapeutic protocol. This is also particularly important in the development of new therapeutic agents to be effective against known resistant variants of HBV and also when cross resistance develops within a family of chemically related anti-viral agents.

HBV baculovirus mediated HBV replication is a transient system and does not require integration of the HBV viral genome. This system was recently described by Delaney *et al.* (13, 14). The HBV baculovirus system has a number of advantages over standard transient transfection systems and cell lines expressing HBV.

In the study of HBV replication and the development of therapeutic agents directed against HBV, some cell lines have been developed which are capable of expressing HBV DNA. However, these cell lines were developed using HBV DNA sequences under the control of heterologous promoters or heterologous regulatory sequences which are unlikely to mimic the situation in a naturally infected cell.

Furthermore, cell lines commonly used to study HBV contain multiple copies of integrated HBV DNA. Hepadnavirus genomes are maintained in the nucleus of infected cells *in vivo* as a pool of episomal, covalently closed circular (CCC) DNA molecules. Although the integration of HBV DNA in human liver has been reported, it is not an obligatory part of the HBV lifecycle and integration is not required for HBV replication. In addition, when integrated HBV DNA is found, it is frequently rearranged and is often transcriptionally silent. Because HBV expressing cell lines contain stably integrated HBV DNA, viral gene expression and replication is continuous; therefore, it is not possible to experimentally control the time or conditions under which these processes are initiated. Stable HBV expressing cell lines contain fixed numbers of integrated typically head-to tail orientated HBV genomes and, as such, HBV gene expression and replication levels cannot be regulated and are restricted to the number of integrated copies which each cell line contains. Consequently, it is not possible to study the effects of increasing or decreasing the copy number of integrated HBV genomes without transfecting the cell line and/or selecting new cell lines.

HBV baculovirus infection, even at high multiplicities, is not toxic to cells such as HepG2 or Huh-7. HBV expression can be enhanced or prolonged in a population of HBV baculovirus infected cells simply by superinfection of the cultures.

One major difference between baculovirus-mediated gene transfer of HBV to HepG2 cells and stably transfected cell lines is the ability to synchronously initiate the replication process. In a stably transfected HBV cell line, such as a derivative of the HepG2 cell line referred to as "2.2.15", each cell contains virus at all phases of the replication cycle. In contrast, HBV baculovirus infection can be used to synchronously start HBV replication in, for example, HepG2 cells because these cells contain no viral products before infection. In HBV baculovirus infected HepG2 cells, it is possible to follow the time course for secretion of both HBsAg and HBeAg with time after infection using the appropriate recombinant HBV baculovirus.

There is a need, therefore, to develop a baculovirus system to screen for specific HBV variants having altered sensitivities to particular agents.

SUMMARY OF THE INVENTION

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Nucleotide and amino acid sequences are referred to by a sequence identifier, i.e. <400>1, <400>2, etc. A sequence listing is provided after the claims.

The present invention relates generally to an assay for detecting a variant HBV which exhibits altered sensitivity to agents. In particular, the assay comprises the use of a baculovirus system to screen for sensitivities of HBV variants to particular agents. The agents contemplated herein include chemical agents such as nucleotide and nucleoside analogues and non-nucleoside analogues, immunological agents such as antibodies and cytokines as well as other therapeutic molecules. These agents generally but not exclusively have a known effect against a reference HBV such as inhibiting or reducing HBV infection, replication and/or assembly or release of virus or virus-like particles. The assay generally comprises detecting a variant HBV which exhibits an altered sensitivity to an agent by first generating a genetic construct comprising a replication competent amount of the genome from the HBV variant contained in or fused to an amount of baculovirus genome capable of infecting cells. Before, during or after the cells are infected, an agent to be tested is brought into contact with the cells. There is an optional further step where the cells are again infected with the same construct or a genetic construct comprising the genome of an HBV wild-type or other HBV variant. The cells are then cultured for a time and under conditions sufficient for the variant HBV to replicate, express genetic sequences and/or assembly and/or release virus or virus-like particles if resistant to the agent. The cells, cell lysates or culture supernatant fluid are then subjected to viral- or viral-component-detection means to determine whether or not the HBV variant has replicated, expressed genetic material and/or assembly and/or has been released in the presence of an

agent. The presence or absence of the detectable components provides an indication of resistance or sensitivity to the agent.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A is a diagrammatic representation showing overlapping genome of HBV.

Figure 1B is a diagrammatic representation showing examples of the major mutations detected in the precore, envelope and polymerase genes.

Sub a1

Figure 2 is a representation of the amino acid consensus sequence from HBV DNA polymerase proteins encompassing regions which are conserved in the RNA polymerase protein. These regions are shown as domains A-E and are underlined. In the consensus sequence the M in the YMDD motif is designated as amino acid number 550. The amino acids which are subject to mutation during 3TC and/or FCV treatment are shown in bold. An asterisk (*) indicates greater than three amino acid possibilities at this position of the consensus sequence. The HBsAg major hydrophilic region containing the neutralisation domain is indicated by a double line and the polymerase mutations which alter the HBsAg are indicated in italics.

Sub a2

Figure 3 is a representation of the nucleotide sequence from various strains of HBV encoding the surface antigen. The amino acid sequence of the surface antigen beginning at amino acid 108 is shown above the nucleotide sequence.

Figure 4A is a diagrammatical representation of pBBHVB1.28 carrying the HBV 1.28 genome.

Figure 4B is a diagrammatical representation of pBBHVB1.5 carrying the HBV 1.5 genome.

Sub a3

Figure 5A is the representation of the nucleotide sequence of HBV 1.28 genome.

Sub a4

Figure 5B is the representation of the nucleotide sequence of HBV 1.5 genome.

Figures 6A and 6B are graphical representations of antiviral testing performed with wild-type HBV baculovirus using A, 3TC; B, PMEA.

Figure 6C is a photographic representation of a Southern blot showing the effect of PCV on wild-type HBV baculovirus.

Figures 7A to 7C are graphical representations of antiviral testing performed with L526M HBV baculovirus using A, 3TC; B, PMEA; C, PCV.

Figures 8A to 8C arc graphical representations of antiviral testing performed with L526M/M550V HBV baculovirus using A, 3TC; B, PMEA; C, PCV.

Figures 9A to 9C are graphical representations of antiviral testing performed with M550I HBV baculovirus using A, 3TC; B, PMEA; C, PCV.

Figure 10 is a graphical representation showing the competition of radio-labelled [α^{32}P]-dCTP by cold dCTP using the endogenous polymerase assay with particles prepared from baculovirus infected cells.

Figures 11A to 11C are photographic representations showing Southern blot of the intracellular and extracellular HBV DNA production from HepG2 cells transduced with wildtype (WT) and precore (G1896A) recombinant HBV-baculovirus exposed to increasing concentrations of (A) adefovir, (B) lamivudine and (C) penciclovir. IC, intracellular; EC, extracellular; RC, relaxed circular HBV DNA; DS, linear double-stranded HBV DNA; SS, single-stranded HBV DNA.

Figure 12 is a photographic representation showing Southern blot of intracellular and extracellular HBV DNA production from HepG2 cells transduced with various recombinant HBV-baculovirus M550I, precore/M550I, L526M/M550V and precore/L526M/M550V.

Figures 13A to 13F are photographic representations showing Southern blot of intracellular and extracellular HBV DNA production from HepG2 cells transduced with recombinant HBV-baculovirus [M550I and precore/M550I (Figures 13A, 13C, 13E) L526M/M550V and precore/L526M/M550V (Figures 13B, 13D, 13F)] exposed to various concentrations of adefovir, or lamivudine, or penciclovir. The extracellular virus production from cells transduced with L526M/M550V was too low to be measured. IC, intracellular; EC, extracellular; RC, relaxed circular HBV DNA; DS, linear double-stranded HBV DNA; SS, single-stranded HBV DNA.

The following abbreviations are used in the subject specification:

Abbreviations	Definitions
3TC, LAM	(-)-b-L-2',3'-dideoxy-3'-thiacytidine
PMEA	9-(2-phosphonylmethoxyethyl)adenine
PCV	9-[4-hydroxy-3-(hydroxymethyl)but-1-yl]guanine
YMDD	Amino acid motif from HBV DNA polymerase; Try Met Asp Asp; the Met is residue 550
HBV	Hepatitis B virus
Xaa	Any amino acid
Pre-S1, Pre-S2, S	Viral envelope genes; S encodes HBsAg
HBsAg	HBV surface antigen
CCC DNA	Covalently closed circular DNA
moi	Multiplicity of infection
pfu	plaque forming units
DNA	Deoxyribonucleic acid
HBeAg	hepatitis B e antigen
RNA	Ribonucleic acid
PCR	Polymerase Chain Reaction
ELISA	Enzyme Linked Immunosorbent Assay
p.i.	Post infected
P	Polymerase gene
IC	intracellular
EC	extracellular
RC	Relaxed circular HBV DNA
DS	Linear double stranded HBV DNA
SS	Single-stranded HBV DNA
LDH	lactate dehydrogenase
FCV	Famciclovir

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides an assay using variant HBV baculovirus to screen for the sensitivities of HBV variants to particular agents. These agents generally but not exclusively have a known effect against a reference HBV such as inhibiting or reducing HBV infection, replication and/or assembly and/or release of virus or virus-like particles. The present invention provides an assay to screen for the sensitivities of HBV variants to particular agents. These agents generally but not exclusively have a known effect against a reference HBV such as inhibiting or reducing HBV infection, replication and/or assembly and/or release of virus or virus-like particles.

The present invention is predicated in part on the identification of HBV variants which have altered sensitivity to agents which would, under standard conditions, have a particular effect or absence of effect on a reference HBV.

Generally, although not exclusively, the agent inhibits or reduces HBV infection, replication and/or assembly and/or release of virus or virus-like particles. The assay determines whether the agent has the same effect on a particular variant HBV. The assay is also useful in determining the extent of cross resistance within a group of chemically or functionally related anti-viral agents. In one embodiment, the variant HBV is an escape mutant. The present invention extends, however, to the case where an agent has little effect on a reference HBV but is effective against the variant HBV. For the present purposes, a "reference" HBV is conveniently regarded as a "wild-type" HBV.

The term "variant" is used in its broadest context and includes mutants, derivatives, modified and altered forms of an HBV relative to a reference HBV. A variant generally contains a single or multiple nucleotide substitution, addition and/or deletion or truncation mutation in the viral genome and a corresponding single or multiple amino acid substitution, addition and/or deletion or truncation in a viral peptide, polypeptide or protein.

Accordingly, one aspect of the present invention contemplates a method for detecting a variant HBV which exhibits an altered sensitivity to an agent, said method comprising:-

generating a genetic construct comprising a replication competent amount of the genome from said variant HBV contained in or fused to an amount of a baculovirus genome capable to infect cells, and then infecting said cells with said construct;

contacting said cells, before, during and/or after infection, with the agent to be tested;

optionally further infecting said cells with the same genetic construct or a genetic construct comprising the genome of HBV wild-type or another HBV variant;

culturing said cells for a time and under conditions sufficient for the variant HBV to replicate, express genetic sequences and/or assemble and/or release virus or virus-like particles if resistant to said agent; and

subjecting the cells, cell lysates or culture supernatant fluid to viral- or viral-component-detection means to determine whether or not the variant virus has replicated, expressed genetic material and/or assembled and/or been released in the presence of said agent.

Preferably, the altered sensitivity includes an effect on viral infection, replication and/or assembly and/or release of virus or virus-like particles or an effect on intermediary steps during the processes of infection, replication, assembly and/or release. In a particularly preferred embodiment, the identification of whether the HBV variant is resistant to an agent is determined. Resistance to an agent includes resistance to two or more chemically or functionally related agents as may occur during the development of cross resistance.

Accordingly, another aspect of the present invention provides a method for detecting a variant HBV which is capable of infecting, replicating, assembly and/or release in the

presence of an agent which inhibits or reduces infection, replication, assembly and/or release of a reference HBV said method comprising:-

generating a genetic construct comprising a replication competent amount of said variant HBV genome contained in or fused to an amount of a baculovirus genome capable to infect cells and then infecting cells with said construct;

contacting said cells, before and/or during and/or after infection, with the agent to be tested;

optionally further infecting said cells with the same genetic construct or a genetic construct comprising the genome of HBV wild-type or another HBV variant;

culturing said cells for a time and under conditions sufficient for the variant HBV to replicate, express genetic material and/or assemble and/or release virus or virus-like particles if resistant to said agent; and

subjecting the cells or cell lysates to viral- or viral component-detection means to determine whether or not the variant virus has replicated, expressed genetic material or assembled and/or been released in the presence of said agent.

The optional step referred to above encompasses testing of the effects of co-infection by the same or other HBVs.

Accordingly, another aspect of the present invention provides a method for detecting a variant HBV which is capable of infecting, replicating, assembly and/or release in the presence of an agent which inhibits or reduces infection, replication, assembly and/or release of a reference HBV said method comprising:-

generating a genetic construct comprising a replication competent amount of said variant HBV genome contained in or fused to an amount of a baculovirus genome capable to infect cells and then infecting cells with said construct;

contacting said cells, before and/or during, and/or after infection, with the agent to be tested;

optionally further infecting said cells with the same genetic construct or a genetic construct comprising the genome of HBV wild-type or another HBV variant at one or more times after the initial infection;

culturing said cells for a time and under conditions sufficient for the variant HBV to replicate, express genetic material and/or assemble and/or release virus or virus-like particles if resistant to said agent; and

subjecting the cells or cell lysates to viral- or viral component-detection means to determine whether or not the variant virus has replicated, expressed genetic material or assembled and/or been released in the presence of said agent.

The optional step referred to above encompasses testing of the effects of superinfection by the same or other HBVs.

The detection of HBV or its components in cells, cell lysates and culture supernatant fluid may be by any convenient means. For example, total HBV DNA or RNA may be determined, replicative intermediates may be detected or HBV-specific products or gene transcripts may be determined. Suitable assay means includes PCR, nucleic acid hybridization protocols such as northern blots and Southern blots and antibody procedures such as ELISA and Western blot may be employed.

An example of an HBV variant of the present invention is a variant obtained following selective pressure in the clinical setting. One form of selective pressure is chemical

pressure (e.g. *via* nucleoside analogues) directed to the HBV DNA polymerase which selects for a mutation in the gene encoding HBV DNA polymerase. Due to the overlapping nature of the HBV genome, a corresponding mutation may also occur in the gene encoding HBsAg (see Figure 1B). Accordingly, a mutation in one or more nucleotides encoding HBV DNA polymerase may have an effect on the nucleotide sequence encoding HBsAg.

A viral variant may, in accordance with the present invention, carry a mutation only in the DNA polymerase or the surface antigen or may carry a mutation in both genes. The term "mutation" is to be read in its broadest context and includes a silent mutation not substantially affecting the normal function of the DNA polymerase or surface antigen or may be an active mutation having the effect of selection of nucleoside analogue resistance or a vaccine escape mutant phenotype. Where multiple mutations occur in accordance with the present invention or where multiple phenotypes result from a single mutation, at least one mutation must be active or the virus must exhibit at least one altered phenotype such as nucleoside analogue resistance or reduced immunological interactivity of anti-HBs to the surface antigen of a reference HBV.

The present invention extends to assaying any HBV mutant carrying a single or multiple substitution, addition and/or deletion or truncation in the amino acid sequence of the catalytic region of the HBV DNA polymerase as compared to the amino acid sequence set forth in Formula I which is considered herein to define a reference HBV:-

FORMULA I

$$S\ Z_{1}\ L\ S\ W\ L\ S\ L\ D\ V\ S\ A\ A\ F\ Y\ H\ Z_{2}\ P\ L\ H\ P\ A\ A\ M\ P\ H\ L\ L\ Z_{3}\ G\ S\ S\ G\ L\ Z_{4}\ R\ Y\ V\ A\ R\ L\ S\ S\ Z_{5}\ S\ Z_{6}\ Z_{7}\ X\ N\ Z_{8}\ Q\ Z_{9}\ Z_{10}\ X\ X\ X\ Z_{11}\ L\ H\ Z_{12}\ Z_{13}\ C\ S\ R\ Z_{14}\ L\ Y\ V\ S\ L\ Z_{15}\ L\ L\ Y\ Z_{16}\ T\ Z_{17}\ G\ Z_{18}\ K\ L\ H\ L\ Z_{19}\ Z_{20}\ H\ P\ I\ Z_{21}\ L\ G\ F\ R\ K\ Z_{22}\ P\ M\ G\ Z_{23}\ G\ L\ S\ P\ F\ L\ L\ A\ Q\ F\ T\ S\ A\ I\ Z_{24}\ Z_{25}\ Z_{26}\ Z_{27}\ Z_{28}\ R\ A\ F\ Z_{29}\ H\ C\ Z_{30}\ Z_{31}\ F\ Z_{32}\ Y\ M^{*}\ D\ D\ Z_{33}\ V\ L\ G\ A\ Z_{34}\ Z_{35}\ Z_{36}\ Z_{37}\ H\ Z_{38}\ E\ Z_{39}\ L\ Z_{40}\ Z_{41}\ Z_{42}\ Z_{43}\ Z_{44}\ Z_{45}\ Z_{46}\ L\ L\ Z_{47}\ Z_{48}\ G\ I\ H\ L\ N\ P\ Z_{49}\ K\ T\ K\ R\ W\ G\ Y\ S\ L\ N\ F\ M\ G\ Y\ Z_{50}\ I\ G$$

wherein:

X is any amino acid;
Z_1 is N or D;
Z_2 is I or P;
Z_3 is I or V;
Z_4 is S or D;
Z_5 is T or N;
Z_6 is R or N;
Z_7 is N or I;
Z_8 is N or Y or H;
Z_9 is H or Y;
Z_{10} is G or R;
Z_{11} is D or N;
Z_{12} is D or N;
Z_{13} is S or Y;
Z_{14} is N or Q;
Z_{15} is L or M;
Z_{16} is K or Q;
Z_{17} is Y or F;
Z_{18} is R or W;
Z_{19} is Y or L;
Z_{20} is S or A;
Z_{21} is I or V;
Z_{22} is I or L;
Z_{23} is V or G;
Z_{24} is C or L;
Z_{25} is A or S;
Z_{26} is V or M;
Z_{27} is V or T;

Z_{28} is R or C;
Z_{29} is F or P;
Z_{30} is L or V;
Z_{31} is A or V;
Z_{32} is S or A;
Z_{33} is V or L or M;
Z_{34} is K or R;
Z_{35} is S or T;
Z_{36} is V or G;
Z_{37} is Q or E;
Z_{38} is L or S or R;
Z_{39} is S or F;
Z_{40} is F or Y;
Z_{41} is T or A;
Z_{42} is A or S;
Z_{43} is V or I;
Z_{44} is T or C;
Z_{45} is N or S;
Z_{46} is F or V;
Z_{47} is S or D;
Z_{48} is L or V;
Z_{49} is N or Q;
Z_{50} is V or I; and
M^* is amino acid 550.

Accordingly, another aspect of the present invention provides an assay for detecting an HBV variant having an altered sensitivity to an agent, said variant HBV comprising a single or multiple amino acid substitution, addition and/or deletion or truncation compared to the amino acid sequence set forth in Formula I and wherein the variant HBV is selected for by a nucleoside analogue of the HBV DNA polymerase said method comprising:-

generating a genetic construct comprising a replication competent amount of the genome from said variant HBV contained in or fused to an amount of a baculovirus genome capable to infect cells and infecting said cells with said construct;

contacting said cells, before and/or during, and/or after infection, with the agent to be tested;

optionally further infecting said cells with the same genetic construct or a genetic construct comprising the genome of HBV wild-type or another HBV variant;

culturing said cells for a time and under conditions sufficient for the variant HBV to replicate, express genetic sequences and/or assemble and/or release virus or virus-like particles if resistant to said agent; and

subjecting the cells, cell lysates or culture supernatant fluid to viral- or viral-component-detection means to determine whether or not the variant virus has replicated, expressed genetic material or assembled and/or been released in the presence of said agent.

In a related embodiment the present invention is directed to an assay for an HBV variant, in which the HBV variant exhibits reduced sensitivity to an agent which otherwise inhibits or reduces infection, replication or assembly and/or release by a reference HBV, said variant HBV comprising a single or multiple amino acid substitution, addition and/or deletion or truncation compared to the amino acid sequence set forth in Formula I, said method comprising:-

generating a genetic construct comprising a replication competent amount of said variant HBV genome contained in or fused to an amount of a baculovirus genome capable to infect cells and then infecting cells with the construct;

contacting said cells, before, and/or during, and/or after infection, with the agent to be tested;

optionally further infecting said cells with the same genetic construct or a genetic construct comprising the genome of HBV wild-type or another HBV variant;

culturing said cells for a time and under conditions sufficient for the variant HBV, express genetic material and/or assemble and/or release virus or virus-like particles if resistant to said agent; and

subjecting the cells or cell lysates to viral- or viral component-detection means to determine whether or not the variant virus has replicated, expressed genetic material or assembled and/or been released in the presence of said agent.

Another HBV variant contemplated by the present invention is in the precore or the basal core promoter sequences. Precore mutations contemplated by the present invention include those listed in Table 1 and include. Particular precore gene and basal core promoter (BCP) mutations include A1814T, C1856T, G1896A, G1897A, G1898A, G1899A, G1896A/ G1899A, A1762T/ G1764A, T1753C, G1757A and C1653T (where the numbering is from the unique *Eco*R1 site in HBV).

Accordingly, another aspect of the present invention provides an assay for an HBV variant with an altered sensitivity to an agent, said variant HBV comprising a nucleotide sequence containing a single or multiple nucleotide substitution, addition and/or deletion to the precore nucleotide sequence, said method comprising:-

generating a genetic construct comprising a replication competent amount of the genome from said variant HBV contained in or fused to an amount of a baculovirus genome capable to infect cells and infecting said cells with said construct;

contacting said cells, before and/or during, and/or after infection, with the agent to be tested;

optionally further infecting said cells with the same genetic construct or a genetic construct comprising the genome of HBV wild-type or another HBV variant;

culturing said cells for a time and under conditions sufficient for the variant HBV to replicate, express genetic sequences or assemble if resistant to said agent; an d

subjecting the cells, cell lysates or culture supernatant fluid to viral- or viral-component-detection means to determine whether or not the variant virus has replicated, expressed genetic material or assembled in the presence of said agent.

Preferably, the HBV variant according to this aspect of the present invention has a precore gene or a BCP mutation as listed in Table 1. In a particularly preferred embodiment, the precore or a BCP mutation is selected from A1814T, C1856T, G1896A, G1897A, G1898A, G1899A, G1896A/G1899A, A1762T/ G1764A, T1753C, G1757A and C1653T (where the numbering is from the unique *Eco*R1 site in HBV).

In a related embodiment, the present invention contemplates an assay for an HBV variant which exhibits reduced sensitivity to an agent which otherwise inhibits or reduces infection, replication or assembly and/or release by a reference HBV, said variant HBV comprising a nucleotide sequence comprising a single or multiple nucleotide substitution, addition and/or deletion to the precore nucleotide sequence, said method comprising:-

generating a genetic construct comprising a replication competent amount of the genome from said variant HBV contained in or fused to an amount of a baculovirus genome capable to infect cells and infecting said cells with said construct;

contacting said cells, before, and/or during, and/or after infection, with the agent to be tested;

optionally further infecting said cells with the same genetic construct or a genetic construct comprising the genome of HBV wild-type or another HBV variant;

culturing said cells for a time and under conditions sufficient for the variant HBV to replicate, express genetic sequences or assemble if resistant to said agent; and

subjecting the cells, cell lysates or culture supernatant fluid to viral- or viral-component-detection means to determine whether or not the variant virus has replicated, expressed genetic material or assembled in the presence of said agent.

In another embodiment, the HBV variant comprises a precore mutation together with a mutation in the DNA polymerase or the corresponding mutation in the hepatitis B surface antigen (HBsAg). Preferred mutations according to this embodiment result from resistance to lamivudine (LAM) such as but are limited to a precore mutation together with L526M+M550V [M195I] or a precore mutation together with M550I [W196L, W196S or W196STOP]. The mutations given in parenthesis are the corresponding HBsAg mutations following a mutation in the HBV DNA polymerase gene. Preferred precore gene and BCP mutations are A1814T, C1856T, G1896A, G1897A, G1898A, G1899A, G1896A/G1899A, A1762T/G1764A, T1753C, G1757A, G1653T (Table 1).

Accordingly, another aspect of the present invention provides an assay for an HBV variant with an altered sensitivity to LAM or another agent, said variant HBV comprising a nucleotide sequence comprising a single or multiple nucleotide substitution, addition and/or deletion to the precore nucleotide sequence, and the DNA polymerase gene and optionally the overlapping HBsAg nucleotide sequence said method comprising:-

generating a genetic construct comprising a replication competent amount of the genome from said variant HBV contained in or fused to an amount of a baculovirus genome capable to infect cells and infecting said cells with said construct;

contacting said cells, before and/or during, and/or after infection, with the agent to be tested;

optionally further infecting said cells with the same genetic construct or a genetic construct comprising the genome of HBV wild-type or another HBV variant;

culturing said cells for a time and under conditions sufficient for the variant HBV to replicate, express genetic sequences or assemble if resistant to said agent; and

subjecting the cells, cell lysates or culture supernatant fluid to viral- or viral-component-detection means to determine whether or not the variant virus has replicated, expressed genetic material or assembled in the presence of said agent.

In a related embodiment, the present invention contemplates an assay for an HBV variant which exhibits reduced sensitivity to LAM or another agent and which otherwise inhibits or reduces infection, replication or assembly and/or release by a reference HBV, said variant HBV comprising a nucleotide sequence encoding a single or multiple nucleotide substitution, addition and/or deletion to the precore nucleotide sequence, and in the DNA polymerase gene and optionally the HBsAg nucleotide sequence said method comprising:-

generating a genetic construct comprising a replication competent amount of the genome from said variant HBV contained in or fused to an amount of a baculovirus genome capable to infect cells and infecting said cells with said construct;

contacting said cells, before, and/or during, and/or after infection, with the agent to be tested;

optionally further infecting said cells with the same genetic construct or a genetic construct comprising the genome of HBV wild-type or another HBV variant;

culturing said cells for a time and under conditions sufficient for the variant HBV to replicate, express genetic sequences or assemble if resistant to said agent; and

subjecting the cells, cell lysates or culture supernatant fluid to viral- or viral-component-detection means to determine whether or not the variant virus has replicated, expressed genetic material or assembled in the presence of said agent.

Preferably, the HBV variants according to these aspects of the present invention has a precore gene and BCP mutation as listed in Table 1. In a particularly preferred embodiment, the precore or a BCP mutation is selected from A1814T, C1856T, G1896A, G1897A, G1898A, G1899A, G1896A/ G1899A, A1762T/ G1764A, T1753C, G1757A and C1653T (where the numbering is from the unique *Eco*R1 site in HBV). Preferably, the mutation of the DNA polymerase gene [and HBsAg] is L526M+M550V [M195I] or M550I [W196L, W196S or W196STOP].

Another example of a variant to be tested in accordance with the present invention is one with an altered immunological profile. Such a variant would substantially not be affected by a neutralizing immune response directed to a conventional HBV vaccine such as a vaccine comprising a reference HBV or a surface component thereof. Similarly, the variant HBV may substantially not be affected at the infection, replication, assembly or release level or another level during the life cycle of the HBV by an agent which is capable of inhibiting or reducing infection, replication, assembly or release of a reference HBV. The expression "substantially not affected" includes reduced susceptibility to the immune response generated by a vaccine or reduce susceptibility to chemical agents such as nucleoside analogues which target HBV genes such as the DNA polymerase. Due to the overlapping nature of reading frames for DNA polymerase and certain viral surface components, an altered surface component may have a corresponding alteration in the DNA polymerase.

A preferred surface component of the HBV of the present invention is HBsAg. It is proposed in accordance with this aspect of the present invention that the HBsAg of the

HBV variants exhibit an altered immune profile relative to an HBsAg from a reference HBV. For the purposes of the present invention, a reference HBV conveniently comprises an HBsAg with an amino acid sequence substantially as set forth by Norder *et al.* (1) and Stuyver *et al.* (2) which encompasses all known genotypes of HBV (currently A through G). The assay of the present invention can be used to screen for the effect of the altered HBsAg on the sensitivity of a variant HBV to a particular agent.

The present invention extends, therefore, to any single or multiple amino acid substitution, addition and/or deletion or truncation in the amino acid sequence of HBsAg relative to the amino acid sequence set forth in Formula II below as defined by a single or multiple amino acid substitution, addition and/or deletion to the catalytic region of the HBV DNA polymerase set forth above in Formula I.

The amino acid sequence of an HBsAg and which is considered to define a reference HBV is set forth below in Formula II:-

FORMULA II

$M\ X_1\ X_2\ X_3\ X_4\ S\ G\ X_5\ L\ X_6\ P\ L\ X_7\ V\ L\ Q\ A\ X_8\ X_9\ F\ X_{10}\ L\ T\ X_{11}\ I\ X_{12}\ X_{13}\ I\ P\ X_{14}\ S\ L$
$X_{15}\ S\ W\ W\ T\ S\ L\ N\ F\ L\ G\ X_{16}\ X_{17}\ X_{18}\ X_{19}\ C\ X_{20}\ G\ X_{21}\ N\ X_{22}\ Q\ S\ X_{23}\ X_{24}\ S\ X_{25}\ H\ X_{26}\ P$
$X_{27}\ X_{28}\ C\ P\ P\ X_{29}\ C\ X_{30}\ G\ Y\ R\ W\ M\ C\ L\ X_{31}\ R\ F\ I\ I\ F\ L\ X_{32}\ I\ L\ L\ L\ C\ L\ I\ F\ L\ L\ V\ L\ L\ D$
$X_{33}\ Q\ G\ M\ L\ X_{34}\ V\ C\ P\ L\ X_{35}\ P\ X_{36}\ X_{37}\ X_{38}\ T\ T\ S\ X_{39}\ X_{40}\ X_{41}\ C\ X_{42}\ T\ C\ X_{43}\ X_{44}\ X_{45}\ X_{46}$
$Q\ G\ X_{47}\ S\ X_{48}\ X_{49}\ P\ X_{50}\ X_{51}\ C\ C\ X_{52}\ K\ P\ X_{53}\ X_{54}\ G\ N\ C\ T\ C\ I\ P\ I\ P\ S\ X_{55}\ W\ A\ X_{56}\ X_{57}$
$X_{58}\ X_{59}\ L\ W\ E\ X_{60}\ X_{61}\ S\ X_{62}\ R\ X_{63}\ S\ W\ L\ X_{64}LLX_{65}X_{66}\ F\ V\ Q\ X_{67}\ X_{68}\ X_{69}\ X_{70}\ L\ X_{71}\ P$
$X_{72}\ V\ W\ X_{73}\ X_{74}\ X_{75}\ I\ W\ X_{76}\ X_{77}\ W\ X_{78}\ W\ X_{79}\ P\ X_{80}\ X_{81}\ X_{82}\ X_{83}\ I\ X_{84}\ X_{85}\ P\ F\ X_{86}\ P\ L\ L$
$P\ I\ F\ X_{87}\ X_{88}\ L\ X_{89}\ X_{90}\ X_{91}\ I$

wherein:

X_1 is E or G or D;

X_2 is N or S or K;

X_3 is I or T;

X_4 is T or A;

X_5 is F or L;

X_6 is G or R;

X_7 is L or R;

X_8 is G or V;

X_9 is F or C;

X_{10} is L or S or W;

X_{11} is R or K;

X_{12} is L or R;

X_{13} is T or K;

X_{14} is Q or K;

X_{15} is D or H;

X_{16} is G or E or A;

X_{17} is S or A or V or T or L;

X_{18} is P or T;

X_{19} is V or R or T or K or G;

X_{20} is L or P;

X_{21} is Q or L or K;

X_{22} is S or L;

X_{23} is P or Q;

X_{24} is T or I;

X_{25} is N or S;

X_{26} is S or L;

X_{27} is T or I;

X_{28} is S or C;

X_{29} is I or T;

X_{30} is P or A;

X_{31} is R or Q;

X_{32} is F or C;

X_{33} is Y or C;

X_{34} is P or H or S;

X_{35} is I or L;

X_{36} is G or R;

X_{37} is S or T;

X_{38} is T or S;

X_{39} is T or V or A;

X_{40} is G or E or Q;

X_{41} is P or A or S;

X_{42} is K or R;

X_{43} is T or M;

X_{44} is T or I or S or A;

X_{45} is P or T or A or I or L;

X_{46} is A or V;

X_{47} is N or T;

X_{48} is M or K or L;

X_{49} is F or Y or I;

X_{50} is S or Y;

X_{51} is C or S;

X_{52} is T or I or S;

X_{53} is T or S;

X_{54} is D or A;

X_{55} is S or T;

X_{56} is F or L;

X_{57} is A or G or V;

X_{58} is K or R or T;

X_{59} is Y or F;

X_{60} is W or G;

X_{61} is A or G;

X_{62} is V or A;

X_{63} is F or L;

X_{64} is S or N;

X_{65} is V or A;

X_{66} is P or Q;

X_{67} is W or C or S;

X_{68} is F or C;

X_{69} is V or D or A;

X_{70} is G or E;

X_{71} is S or F;

X_{72} is T or I;

X_{73} is L or P;

X_{74} is S or L;

X_{75} is A or V;

X_{76} is M or I;

X_{77} is M or I;

X_{78} is Y or F;

X_{79} is G or E;

X_{80} is S or N or K;

X_{81} is L or Q;

X_{82} is Y or F or H or C;

X_{83} is S or G or N or D or T;

X_{84} is V or L;

X_{85} is S or N;

X_{86} is I or M or L;

X_{87} is F or C;

X_{88} is C or Y;

X_{89} is W or R;

X_{90} is V or A; and

X_{91} is Y or I or S.

Accordingly, another aspect of the present invention provides a method for detecting a variant HBV having an altered sensitivity to an agent, the said variant HBV comprising a surface antigen having an amino acid sequence with a single or multiple amino acid

substitution, addition and/or deletion or a truncation compared to a surface antigen from a reference HBV such that an antibody generated to the reference surface antigen exhibits altered capacity for neutralizing said HBV variant said method comprising:

generating a genetic construct comprising a replication competent amount of the genome from said variant HBV contained in or fused to an amount of a baculovirus genome capable to infect cells and then infecting said cells with said construct;

contacting said cells, before, and/or during, and/or after infection, with the agent to be tested;

culturing said cells for a time and under conditions sufficient for the variant HBV to replicate, express genetic sequences or assemble if resistant to said agent; and

subjecting the cells, cell lysates or culture supernatant fluid to viral- or viral-component-detection means to determine whether or not the variant virus has replicated, expressed genetic material or assembled in the presence of said agent.

The amino acid sequence of the HBsAg of the reference HBV is as set forth in Formula II above.

Generally, the method of the present invention is capable of detecting an HBV variant which may be regarded as an escape mutant which it is substantially incapable of being adversely effected by chemical therapy directed against the HBV polymerase or vaccine therapy directed against the surface antigen. The term "escape" mutant also encompasses reduced susceptibility to chemical or vaccine therapy directed to the reference HBV.

More particularly, another aspect of the present invention provides a method for detecting a variant HBV which is capable of replicating in the presence of an agent which inhibits or reduces replication of a reference HBV, said reference HBV comprising a surface antigen having an amino acid sequence with a single or multiple amino acid substitution, addition

and/or deletion or a truncation compared to a surface antigen from a reference HBV such that an antibody generated to the reference surface antigen exhibits altered capacity for neutralizing said HBV variant said method comprising:-

generating a genetic construct comprising a replication competent amount of said variant HBV genome contained in or fused to an amount of a baculovirus genome capable to infect cells and then infecting cells with the construct;

contacting said cells, before and/or during, and/or after infection, with the agent to be tested;

culturing said cells for a time and under conditions sufficient for the variant HBV to replicate, express genetic material or assemble if resistant to said agent; and

subjecting the cells or cell lysates to viral- or viral component-detection means to determine whether or not the variant virus has replicated, expressed genetic material or assembled in the presence of said agent.

The present invention also extends to changes in the HBsAg following immunological selection based on vaccines comprising HBsAg or a derivative thereof or an HBV comprising same and wherein the HBsAg comprises an amino acid sequence substantially as set forth in Formula II.

An "agent" therefore extends to a chemical agent (e.g. nucleotide and nucleoside analogues and non-nucleoside analogues), an immunological agent (e.g. antibodies or cytokines) or other therapeutic molecule. One group of agents contemplated by the present invention are non-nucleoside analogue reverse transcriptase inhibitors (such as but not limited to AT-61 a phenylpropenamide derivative; 16) and non-nucleoside analogue DNA dependent DNA polymerase inhibitors. A group of nucleoside analogues contemplated herein comprises 3TC, PMEA and PCV and related molecules.

Reference to an altered immunological profile in accordance with the present invention in relation to the surface antigen includes reference to an altered humoral or T cell response. Examples of an altered immunological profile include altered specificity to antibodies, altered amino acid sequences of an epitope or within the "a" determinant, an altered capacity to induce proliferation of T cells primed to an HBsAg from a reference HBV. Preferably, the altered immunological profile means that neutralizing antibodies which are capable of substantially neutralising or otherwise reducing serum or blood levels of the reference HBV are substantially incapable of or exhibit reduced capacity to neutralize and/or clear the variant HBV.

The HBsAg mutations of the present invention may also be defined in terms of a corresponding mutation in the HBV DNA polymerase. A mutation in the HBV DNA polymerase may be in amino acids 421-431, 426-436, 431-441, 436-446, 441-451, 446-456, 451-461, 456-466, 461-471, 466-476, 471-481, 476-486, 481-491, 486-496, 491-501, 496-506, 501-511, 506-516, 511-521, 516-526, 521-531, 526-536, 531-541, 536-546, 541-551, 546-556, 551-561, 556-566, 561-571, 566-576, 571-581, 576-586, 581-591, 586-596, 591-601, 596-601 (referring to the amino acid numbering in Figure 2).

Preferred HBV DNA polymerase mutations include L426I/V, L428I/V, Q476, N480G, N485K, K495R, R499Q, G499E, W499Q, F512L, I515L, V519L, L526M, M550V, M550I, V553I, S565P. Useful multiple mutants include L526M/M550I, L526M/M550V, V519L/L526M/M550V and V519L/L526M/M550I.

Preferred mutations in the amino acid sequence of HBsAg are amino acid substitutions, deletions and/or additions or truncations in amino acids 1-10, 5-15, 10-20, 15-25, 20-30, 25-35, 30-40, 35-45, 40-50, 45-55, 50-60, 55-65, 60-70, 65-75, 70-80, 75-85, 80-90, 85-95, 90-100, 95-105, 100-110, 105-115, 110-120, 115-125, 120-130, 125-135, 130-140, 135-145, 140-150, 145-155, 150-160, 155-165, 160-170, 165-175, 170-180, 175-185, 180-190, 185-195, 190-200, 195-205, 200-210, 205-215, 210-220, 215-225, 220-226 (referring to the numbering of Formula II) of HBsAg. Particularly useful mutations are G112R, T123P

Y/F134S, D144E, G145R, A157D, E164D, F170L, M195I, W196L, W196S, W196STOP, M198I, W199S, S204T and S210R. The term "stop" means a stop codon.

Even more preferred mutations are D144E, G145R, A157D, E164D, M195I, W196L, W196S, W196STOP, M198I, W199S and S210R.

Particularly preferred precore gene and BCP mutations are selected from A1814T, C1856T, G1896A, G1897A, G1898A, G1899A, G1896A/ G1899A, A1762T/ G1764A, T1753C, G1757A and C1653T (where the numbering is from the unique *Eco*R1 site in HBV).

The present invention extends to combinations of two or more of the above mutations such as but not limited to a precore mutation and a mutation in the DNA polymerase and optionally the overlapping HBsAg nucleotide sequences resulting in, for example, reduce sensitivity to LAM. Examples of the latter include but are not limited to a precore mutation such as G1896A with L526M+M550V [M195I] or M550I [W196L, W196S or W196STOP].

The altered HBsAg molecules of the HBV variants of the present invention may also be defined at the nucleotide level. The nucleotide sequence encoding the HBsAg from a reference HBV is set forth below in Formula III:-

FORMULA III

A C N_1 A A A C C T N_2 N_3 G G A N_4 G G A A A N_5 T G C A C N_6 T G T A T T C C C A T C C C A T C N_7 T C N_8 T G G G C T T T C G N_9 A A N_{10} A T N_{11} C C T A T G G G A G N_{12} G G G C C T C A G N_{13} C C G T T T C T C N_{14} T G G C T C A G T T T A C T A G T G C C A T T T G T T C A G T G G T T C G N_{15} A G G G C T T T C C C C C A C T G T N_{16} T G G C T T T C A G N_{17} T A T A T G G A T G A T G T G G T N_{18} T T G G G G G C C A A G T C T G T A C A N_{19} C A T C N_{20} T G A G T C C C T T T N_{21} T N_{22}

C C N_{23} C T N_{24} T T A C C A A T T T T C T T N_{25} T G T C T N_{26} T G G G N_{27} A T A C A T T

wherein:

N_1 is A or C;
N_2 is T or A;
N_3 is C or T;
N_4 is C or T;
N_5 is C or T;
N_6 is C or T;
N_7 is A or G;
N_8 is T or C;
N_9 is C or G;
N_{10} is G or A;
N_{11} is T or A;
N_{12} is T or G;
N_{13} is T or C;
N_{14} is C or T;
N_{15} is T or C;
N_{16} is T or C;
N_{17} is T or C;
N_{18} is A or T;
N_{19} is A or G;
N_{20} is T or G;
N_{21} is A or T;
N_{22} is A or G;
N_{23} is T or G;
N_{24} is A or G;
N_{25} is T or C;
N_{26} is T or C; and

N_{27} is T or C.

Accordingly, another aspect of the present invention provides an assay for an HBV variant which an altered sensitivity to an agent, said variant HBV comprising a nucleotide sequence comprising a single or multiple nucleotide substitution, addition and/or deletion to the nucleotide sequence set forth in Formula III and which HBV variant has a surface antigen exhibiting an altered immunological profile relative to a surface antigen defined by Formula II said method comprising:-

generating a genetic construct comprising a replication competent amount of the genome from said variant HBV contained in or fused to an amount of a baculovirus genome capable to infect cells and infecting said cells with said construct;

contacting said cells, before, and/or during, and/or after infection, with the agent to be tested;

optionally further infecting said cells with the same genetic construct or a genetic construct comprising the genome of HBV wild-type or another HBV variant;

culturing said cells for a time and under conditions sufficient for the variant HBV to replicate, express genetic sequences or assemble if resistant to said agent; and

subjecting the cells, cell lysates or culture supernatant fluid to viral- or viral-component-detection means to determine whether or not the variant virus has replicated, expressed genetic material or assembled in the presence of said agent.

Generally, the effective amount of HBV genome required to be inserted into the baculovirus genome is functionally equivalent to but comprises more than 100% of an HBV genome. For example, constructs containing approximately 1.05, 1.1, 1.2, 1.28, 1.3, 1.4, 1.5 and 1.6-1.9, 2.0 and 3.0 times the HBV genome are particularly useful.

Any cells which are capable of infection by baculovirus may be used in the practice of the present invention. The hepatoblastoma cell line, HepG2, or its derivatives, is particularly useful and is capable of *in vitro* cell culture. Huh-7 cells may also be used. Alternatively, any hepatocyte cell line and primary hepatocyte cell culture may be used.

For convenience, a genetic construct comprising an HBV genome and an infection effective amount of baculovirus genome is referred to herein as "HBV baculovirus", "recombinant HBV baculovirus" and "HBV baculovirus vector". Recombinant HBV baculovirus is an efficient vector for the delivery of HBV genetic information to human cells and can be used to initiate HBV gene expression and replication in the cells. HBV transcripts, intracellular and secreted HBV antigens are produced and replication occurs as evidenced by the presence of high levels of intracellular, replicative intermediates and protected HBV DNA in the medium. Viral CCC DNA can be detected indicating that, in this system, HBV core particles are capable of delivering newly synthesized HBV genomes back into the nucleus of infected cells. Strong HBV gene expression can be detected as early as one day post-infected (p.i.) High levels of HBV replicative intermediates, extracellular DNA, and CCC DNA persist through at least 11 days p.i. Endogenous HBV enhancers and promoters may be used to obtain high levels of HBV expression and replication in the cells.

The level of HBV expression and replication in the cells infected with HBV baculovirus can be altered over a considerable range simply by changing the moi.

Furthermore, co-infection or superinfection may occur using the genomes from two or more types of HBV such as two or more HBV variants or a variant and a wild-type strain. In this regard, a number of quasi-species of HBV are generally isolated from subjects infected with HBV which consists of two or more HBV variants, or a variant (or variants) and a wild-type strain. It is important, in some circumstances, to test for these quasi species and to determine the altered sensitivity or resistance of the quasi-species to the therapeutic agents. Nucleic acid or antibody detection systems may be used to detect relative amounts of the different HBVs. This is important for the development of therapeutic protocols

which will need to be effective against HBV variants of a single species as well as multiple HBV variants including the wild type strain.

Reference to "HBV" or its "components" in relation to the detection assay includes reference to the presence of RNA, DNA, antigenic molecules or HBV-specific activities. Conveniently, the assay is conducted quantitively, partially quantitively or qualitively. Most preferably, total HBV RNA or DNA is detected which provides an amount of RNA or DNA in the presence of a particular agent. When the HBV variant is more resistant to a particular agent relative to a wild-type strain, then a graphical representation of total RNA or DNA *versus* concentration of agent is likely to result in a reduced gradient of inhibition and/or an increase in the concentration of agent required before inhibition of RNA or DNA generation.

Another aspect of the present invention provides a method for the detection of polymerase activity from HBV particles isolated from variant HBV baculovirus infected cells and determining the sensitivity of said polymerase to nucleoside triphosphate analogues or non-nucleoside analogues reverse transcriptase inhibitors or non-nucleoside analogues DNA dependent DNA polymerase inhibitors. The HBV particles can be collected from variant HBV cell culture fluid, cell lysates or infected cells. This assay will determine the effect of the said nucleoside triphosphate analogues or non-nucleoside analogues reverse transcriptase inhibitors or non-nucleoside analogues DNA dependent DNA polymerase inhibitors on the reference HBV and variant HBV.

Accordingly, this aspect of the present invention provides a method for detecting a variant HBV comprising DNA polymerase which exhibits an altered sensitivity to an agent, said method comprising:-

generating a genetic construct comprising a replication competent amount of the genome from said variant HBV contained in or fused to an amount of a baculovirus genome capable to infect cells and then infecting said cells with said construct;

optionally further infecting said cells with the same genetic construct or a genetic construct comprising the genome of HBV wild-type or another HBV variant;

culturing said cells for a time and under conditions sufficient for the variant HBV to replicate, express genetic sequences and/or assemble and/or release virus or virus-like particles; and

subjecting the cells, cell lysates or culture supernatant fluid or HBV particles purified therefrom to a HBV DNA polymerase assay in the presence or absence of nucleoside triphosphate analogues or non-nucleoside analogues reverse transcriptase inhibitors or non- nucleoside analogues DNA dependent DNA polymerase inhibitors.

A further aspect of the present invention provides a method for detecting a variant HBV comprising DNA polymerase which exhibits an altered sensitivity to an agent said method comprising:-

generating a genetic construct comprising a replication competent amount of the genome from said variant HBV contained in or fused to an amount of a baculovirus genome capable to infect cells and then infecting said cells with said construct;

optionally contacting said cells, before and/or after infection, with the agent to be tested;

optionally further infecting said cells with the same genetic construct or a genetic construct comprising the genome of HBV wild-type or another HBV variant;

culturing said cells for a time and under conditions sufficient for the variant HBV to replicate, express genetic sequences and/or assemble and/or release virus or virus-like particles if resistant to said agent; and

subjecting the cells, cell lysates or culture supernatant fluid or virus purified therefrom to HBV DNA polymerase assay in the presence or absence of nucleoside triphosphate analogues or non-nucleoside analogues reverse transcriptase inhibitors or non-nucleoside analogues DNA dependent DNA polymerase inhibitors.

Yet a further aspect of the present invention contemplates a method of detecting DNA polymerase activity in the presence of an antiviral agent, said method comprising:-

generating a genetic construct comprising a replication competent amount of a genome from said an HBV capable of producing said DNA polymerase, said genome contained in or fused to an amount of a baculovirus genome capable to infect cells and then infecting said cells with said construct;

contacting said cells, before and/or after infection, with the antiviral agent to be tested;

optionally further infecting said cells with the same genetic construct or a genetic construct comprising the genome of HBV wild-type or another HBV strain;

culturing said cells for a time and under conditions sufficient for the HBV to replicate, express genetic sequences and/or assemble and/or release virus or virus-like particles if resistant to said strain; and

subjecting the cells, cell lysates or culture supernatant fluid or virus purified therefrom to HBV DNA polymerase assay in the presence or absence of nucleoside triphosphate analogues or non-nucleoside analogues reverse transcriptase inhibitors or non-nucleoside analogues DNA dependent DNA polymerase inhibitors.

The present invention is further described by the following non-limiting Examples.

EXAMPLE 1

Overlapping genome of HBV

The overlapping genome of HBV is represented in Figure 1. The gene encoding DNA polymerase (P), overlaps the viral envelope genes, Pre-S1 and Pre-S2, and partially overlaps the X and core (C) genes. The HBV envelope comprises small, middle and large HBV surface antigens. The large protein component is referred to as the HBV surface antigen (HBsAg) and is enclosed by the S gene sequence. The Pre-S1 and Pre-S2 gene sequences encode the other envelope components.

EXAMPLE 2

Amino acid consensus sequence of HBV DNA polymerase

The amino acid consensus sequence for HBV DNA polymerase protein from genotypes A through G is shown in Figure 2.

EXAMPLE 3

Consensus sequence of HBsAg

The nucleotide sequence from various strains of HBV encoding the surface antigen is shown in Figure 3. The amino acid sequence of the surface antigen beginning at amino acid 108 is shown above the nucleotide sequence.

EXAMPLE 4

HBV variants produced by site directed mutagenesis

Table 1 provides a summary of some of the HBV variants produced by site directed mutagenesis.

TABLE 1 *IIBV variants produced by site directed mutagenesis*

Nucleotide analogue selected polymerase mutations	Corresponding surface (S) mutation
1. L426V	No change
2. L426I	No change
3. G499E (B domain)	D144E
4. W499Q (B domain)	G145R
5. F512L (B domain)	A157D
6. V519L (B domain)	E164D
7. L526M (B domain)	No change
8. M550V (C domain)	M195I
9. M550V (C domain)	M196L
10. M550I (C domain)	W196S
11. V553I (C domain)	M198I
12. V55EI (C domain)	W199S
13. S565P	S21OR
Double polymerase mutations	**Corresponding surface (S) mutation**
14. L526M/M550V	M195I
15. L526M/M550I	W196S
16. L426I/M550I	W196S
17. L426V/M550I	W196S
Triple polymerase mutations	**Corresponding surface (S) mutation**
18. V519L/L526M/M550V	E164D/M195I/W196L
19. V519L/L526M/M550I	E164D/W196S
20. L426I/L526M/M550I	W196S
21. L426V/L526M/M550I	W196S
Precore and basal core promoter mutations (number from the unique *Eco*RI site)	
A1814T	
C1856T	
G1896A	
G1897A	
G1898A	
G1899A	
G1896A/G1899A	
A1762T/G1764A	

Precore and basal core promoter mutations (number from the unique *Eco*R1 site) *continued*	
T175C	
G1757A	
G1653T	

EXAMPLE 5

Cell culture

Sf21 insect cells were maintained in supplemented Grace's insect medium further supplemented with 10% v/v heat-inactivated fetal bovine serum (Gibco BRL, Gaithersburg, MD) in humidified incubator at 28°C with CO_2. HepG2 cells were maintained in minimal essential medium supplemented with 10% v/v heat-inactivated fetal bovine serum (MEM-FBS). HepG2 cells were grown in humidified 37°C incubators at 5% v/v CO_2.

EXAMPLE 6

PREPARATION OF BACULOVIRUS TRANSFER VECTOR

A recombinant transfer vector was created by excising a fragment containing the required amount of variant HBV genome construct and cloning it into the multiple cloning region of a baculovirus vector such as pBlueBac4.5 (Invitrogen, Carlsbad, CA). Figures 4A and 4B show a representation of the plasmid encoding the recombinant transfer vector 1.28 and the 1.5 HBV genome construct, respectively. Similar beculovirus transfer vectors may also be constructed from an HBV 1.3 construct. A diagrammatic representation of the recombinant transfer vector HBV 1.3 is shown in Figure 1 of International Patent Application No. PCT/US99/01153 [WO 99/37821]. Analysis of recombinant transfer vector by restriction mapping demonstrated the presence of only one copy of the HBV genome portion in the construct. The nucleotide sequence of the plasmid and the point mutations generated by site directed mutagenesis were confirmed by sequencing using the ABI Prism Big Dye

Terminator Cycle Sequencing Ready Reaction Kit according to the manufacturer's specifications (Perkin Elmer, Cetus Norwalk, CT).

EXAMPLE 7

The sequence of 1.28 HBV genome and the 1.5 HBV genome

The sequence of the 1.28 and the 1.5 HBV genome (Figures 5A and 5B, respectively) were elucidated using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit according to the manufacturer's specifications (Perkin Elmer, Cetus Norwalk, CT).

EXAMPLE 8

Generation of recombinant baculoviruses containing the 1.28, 1.5 or 1.3 HBV construct

Purified recombinant transfer vector and linear AcMNPV baculovirus DNA were co-transfected into Sf21 cells using the BacNBlue transfection kit from Invitrogen (Carlsbad, CA); recombinant viruses were isolated by plaque assay according to the manufacturer's instructions. A series of recombinant viruses were amplified from isolated plaques by infecting 100-mm dishes of Sf21 cells. Viral DNA was extracted from amplified viruses using standard procedures. Purified viral DNA was digested with restriction enzymes and then fractionated by electrophoresis in a 1.0% v/v agarose gel. Southern blotting was performed to determine which virus isolates contained the intact 1.28, 1.5 or 1.3 HBV construct. A Boehringer Mannheim Random Prime DNA Labeling kit (Indianapolis, IN) was used to generate [P^{32}]-radiolabeled probes. A full-length double-stranded HBV genome was used as a template for all radiolabeled probes. Viral DNA sequence was confirmed by PCR amplification of the polymerase catalytic region using the sense primer 5'-GCC TCA TTT TGT GGG TCA CCA TA-3' [<400>1], (nucleotide 1408 to 1430 according to HBV Genebank Accession number M38454) and the antisense primer 5'-TCT CTG ACA TAC TTT CCA AT-3' [<400>2] (nucleotides 2817 to 2798 according to HBV Genebank Accession number M38454). The following primers were utilized for the sequencing of internal regions 5'-TGC ACG ATT CCT GCT CAA-3' [<400>3]

(nucleotides 2345-2362 according to HBV Genbank Accession number M38454) and 5'-TTT CTC AAA GGT GGA GAC AG-3' [<400>4] (nucleotides 1790-1810 according to HBV Genbank Accession number M38454).

EXAMPLE 9

Preparative baculovirus amplification and purification

Baculoviruses were amplified by infecting suspension cultures of Sf21 cells in log phase at a multiplicity of infection (moi) of 0.5 pfu/cell. Infections were allowed to proceed until a majority of the cells in the flasks showed visible signs of infection (four to five days). Virions were concentrated from infected Sf21 medium by centrifugation at 80,000 x g and purified through a 20-60% w/v sucrose gradient. Purified virus was titrated in quadruplicate in Sf21 cells by end-point dilution. An aliquot of each high titre stock was used for DNA extraction. The polymerase gene was amplified and sequenced to confirm the presence of the site-directed mutagenesis as in Example 8.

EXAMPLE 10

Infection of HepG2 cells with recombinant HBV expressing baculovirus

HepG2 cells were seeded at approximately 20-40% confluency and then were grown for 16-24 hours before infection. On the day of infection, triplicate plates of cells were trypsinized, and viable cell number was determined with a hemocytometer using Trypan blue exclusion. Average cell counts were calculated and used to determine the volume of high-titer viral stock necessary to infect cells at the indicated moi. HepG2 cells were washed one time with serum-free MEM to remove traces of serum. Baculovirus was diluted into MEM without serum to achieve the appropriate moi using volumes of 1.0, 0.5, and 0.25 ml to infect 100-mm, 60 mm, and 35-mm dishes, respectively. Baculovirus was adsorbed to HepG2 cells for one hour at 37°C with gentle rocking every 15 minutes to ensure that the inoculum was evenly distributed. The inoculum was then aspirated and HepG2 cells were washed two times with phosphate-buffered saline and refed MEM-FBS with or without various concentrations of agents.

EXAMPLE 11

Analysis of secreted HBV antigens

Detection of hepatitis B surface antigen (HBsAg) and hcpatitis B e antigen (HBeAg) was performed by radioimmunoassay and microparticle enzyme immunoassay using kits purchased from Abbott Laboratories (Abbott Park, IL). Medium from HepG2 cells was collected, centrifuged at 6,000 g to remove cellular debris, transferred to clean tubes, and stored at 20°C until analysis. HBsAg amounts were calculated from a standard curve constructed using known amounts of HBsAg (provided with the kit). HBeAg values are expressed as fold of positive control. Medium samples were diluted appropriately so that radioimmunassay results were within the standard curve (HBsAg) or below positive control values (HBeAg).

EXAMPLE 12

Detection of intracellular replicative intermediates

HBV core particles were isolated from the cytoplasmic fraction of HepG2 cells lysed in 0.5% w/v NP-40. Cytoplasmic extracts were adjusted to 10 mmol/l McC12 and unprotected DNA was removed by an incubation to 500 μg/ml Proteinase K for 1.5 hours at 37°C. Following sequential phenol and chloroform extractions, nucleic acids were recovered by ethanol precipitation. Nucleic acids were resuspended in 50 μl/l TE (10 mmol/l Tris, 1 mmol/l ethylenediaminetetraacetic acid), normalized by OD260, and digested with 100 μg/ml RNase (Boehringer Mannheim, Indianapolis, IN) for one hour at 37°C before analysis by electrophoresis and Southern blotting. A BioRad GS-670 imaging densitometer and the Molecular Analyst software (BioRad, Hecules California) was used to analyze suitable exposures of Southern blots. Densitometry data was fitted to logistic dose response curves using the TableCurve2D software package from Jandel Scientific. Logistic dose response equations were used to calculate IC_{50} and IC_{90} values and co-efficients of variation.

EXAMPLE 13

Extracellular HBV DNA analysis

Conditioned medium was collected from HepG2 cells and subjected to centrifugation at 6,000 g for five minutes to remove cellular debris. HBV particles were precipitated with 10% w/v PEG 8000 and were concentrated by centrifugation at 12,000 xg. Viral pellets were resuspended in 1 ml of MEM-FBS and divided into two aliquots. One set of aliquots was treated with 750 μg/ml Pronase for one hour and then with 500 mg/ml DNase 1 for one hour. Both sets of aliquots were then digested with Proteinase K, and extracted with phenol and chloroform. DNA was precipitated with 0.1 volume of 3 mol/l sodium acetate and 1 volume of isopropanol. Ten micrograms of transfer RNA was added as a carrier during precipitation. Pellets were resuspended in 25 μl of TE and digested with 0.5 mg/ml RNase for one hour. DNA was then analyzed by electrophoresis and Southern blotting.

EXAMPLE 14

3TC, PMEA and PCV treatments

3TC and PCV were a gift from Klaus Esser, SmithKline Beecham, Collegeville, PA. PMEA was obtained from Gilead (Foster City, California). 3TC was resuspended in sterile water, aliquoted, and frozen at -20°C to avoid repeated freezing and thawing of the drug. PMEA was resuspended in sterile water after the pH was adjusted to 7.0 with NaOH. PCV was resuspended in dimethyl sulphoxide. Medium containing 3TC was prepared daily as needed using fresh aliquots of 3TC. In experiments in which 3TC treatment was initiated after viral infection, HepG2 cells were exposed to the indicated concentration of 3TC immediately after infection with HBV baculovirus. In experiments utilizing pretreatment with 3TC, cells were fed medium containing 3TC 16 hours prior to HBV baculovirus infection, HBV baculovirus infection was also carried out in medium containing 3TC, and cells were refed fresh medium containing 3TC immediately after completion of the infection and washing procedures. Treatment with PMEA and PCV were conducted in a similar manner.

EXAMPLE 15

1. Antiviral testing performed with wild-type HBV baculovirus

The dose effect of 3TC and PMEA, on wild-type HBV is shown graphically in Figure 6 (A, B,) and a Southern Blot of intracellular HBV replicative intermediates and extracellular virus produced by HepG2 cells transduced with wild-type HBV-baculovirus in the presence of increasing concentrations of PCV is shown in Figure 6C and the IC_{50} for each antiviral agent is shown in Table 2. 3TC had the most pronounced effect whereas PCV had a modest effect on wild-type HBV replication.

2. Antiviral testing with L526M HBV baculovirus

Figure 7A shows a pronounced resistance of L526M to 3TC compared to the wild-type in Figure 1A in which there was >0.5 log shift in the IC_{50}. No significant change was observed with PMEA or PCV (Figures 7B, 7C).

3. Antiviral testing with L526M/M550V HBV baculovirus

Figure 8A shows that L526M/M550V is completely resistant to 3TC. There was no substantial change to PMEA or PCV (Figures 8B, 8C).

4. Antiviral testing with M550I HBV baculovirus

M550I is completely resistant to 3TC (Figure 9A). The M550I HBV baculovirus had a similar sensitivity to PMEA as the wild-type virus as in Figure 9B.

TABLE 2 *Sensitivity of replication of wt and mutant HBV to inhibition by Lamivudine, Penciclovir and Adefovir*

HBV	Equation Parameters			Correlation (r^2)	Sensitivity Parameters		
	a	*b*	*c*		Inhibition at 1 μM	IC_{50} (μM)	Resistance Factor
				Lamivudine			
wt	185±8	0.004±0.001	1.10±0.07	1.0	99.6	0.009	1
526	102±18	0.027±0.04	0.51±0.27	0.89	85.8	0.03	3
550	186±48	0.35±0.64	0.42±0.26	0.93	26.7	3.8	422
dual	127±10	41.5±31	0.60±0.4	0.90	(-15)**	86.2	9578
				Penciclovir			
wt	101±2	11.2±0.95	1.0±0.06	1.0	7.0	11.5	1
526	100±7	103±12	4.8±7.3	0.93	(-3.0)	103	9
550	124±6	0.0007	—*	0.73	(-24)	1217***	106
dual	111±7	0.002	—*	0.86	(-10)	370	32
				Adefovir			
wt	170±16	0.025±0.001	0.80±0.07	1.0	91.6	0.08	1
526	203±49	0.014±0.01	0.61±0.08	1.0	86.2	0.09	1
550	159±24	0.078±0.05	0.57±0.1	1.0	70.0	0.31	4
dual	155±8	0.039±0.009	0.53±0.02	1.0	77.0	0.16	2

Footnote to Table 2

Measurements were made by assessing the image density of the ds HBV DNA bands in each autoradiograph and expressing the result as a percentage of the mean density of untreated controls.

Logistic dose response (LDR) equations did not accurately describe these data sets; instead the ***a*** and ***b*** parameters are for a single exponential decay equation $y = a^{(-bx)}$.

Values given are means or means ± standard errors. In several cases, low concentrations of inhibitor stimulated HBV replication, reflected by ***a*** values >100%. The percentage inhibition which occurs at a drug concentration of 1 μM has been estimated from each fitted curve plot; negative values (**) represent stimulation of replication relative to controls.

*** Extrapolated value.

The "Resistance Factor" is the factor (to the nearest integer) by which IC_{50} estimated for the mutants differ from the corresponding estimate for wt, calculated by dividing the mutant IC_{50} by wt IC_{50}.

EXAMPLE 16

1. Preparation of HBV particles from HBV baculovirus infected cells

HepG2 cells were infected with 1.5 HBV baculovirus at an moi of 50-200. Starting on day three post infection, conditioned medium was collected from infected cells, centrifuged at 3000 x g to remove cellular debris, and stored frozen at -20°C. HBV particles were concentrated from conditioned medium by ultra-centrifugation in an SW28 rotor at 27,000 rpm for 5 hours. Pelleted virus was resuspended in a small volume phosphate-buffered saline, aliquoted and frozen at -20°C.

2. Endogenous polymerase assay using HBV particles prepared from HBV baculovirus infected cells

Aliquots of virus were thawed and brought to a final concentration of 0.5% NP-40, 2.5 mM Tris pH 7.5, and 6 mM DTT for 10 minutes at room temperature to disrupt the viral envelope. The virus solution was then brought to 150 mM NaCl, 10 mM $MgCl_2$, 20 mM KCL and 10 mM dATP, dGTP and dTTP. 10 mC of ^{32}P-labled dCTP (approximately 0.2 mM) was added and the reaction was incubated at 37°C for 40 minutes to allow the HBV polymerase to extend the viral DNA genome. In order to stop the reaction it was adjusted to 0.5% SDS, 25 mM Tris, and 10 mM EDTA. The reaction mixture was digested with 500 µg/ml of proteinase K overnight at 37°C and HBV DNA was purified from by one extraction with 1:1 phenol:chloroform followed by ethanol precipitation. DNA pellets were resuspended in a small volume of water and electrophoresed through 1% w/v agarose gels for 2 hours. Gels were dried using a Biorad gel dryer and autoradiography of the dried gels was performed to visualize the amount of ^{32}P-labelled dCTP incorporated into HBV genomes by HBV polymerase.

3. *An assay to measure the competition of radio-labelled [α ^{32}P]-dCTP incorporation by cold dCTP using the endogenous polymerase assay using HBV PARTICLES prepared from baculovirus infected cells*

Polymerase assays were performed as described above except that cold dCTP was added to each reaction to compete with the incorporation of labelled dCTP. In separate reactions, concentrations of 0.1, 1, 10, 100, and 1000 mM were added at the same time as labelled dCTP and the polymerase reactions were allowed to proceed. After an extraction of viral DNA and analysis by gel electrophoresis and autoradiography, the amount of labelled DNA from each reaction was quantified by densitometery of the audioradiogram. Densitometry data were fitted to logistic dose response curves using Tablecurve2D software. Figure 10 shows the effect of the competition of cold dCTP on ^{32}P-labelled dCTP.

4. *An assay which can be used to measure the efficacy of an antiviral agents in an endogenous polymerase assay, using HBV particles prepared from baculovirus infected cells*

In an analogous manner, to that described in the above example, the efficacy of antiviral agents such as nucleotide triphosphates and non-nucleoside analogue polymerase inhibitors in inhibiting the catalytic activity of the HBV polymerase can be tested using this assay. The nucleoside triphosphates includes compounds such as 3TC-triphosphate, PMEA-triphosphate or PCV- triphospahte. Polymerase assays can be performed as described in Example 20 part (2), except that various concentrations of an antiviral agent can be added to each reaction to compete with the incorporation of labelled dCTP or another radiolabelled deoxynucleotide triphosphate. In separate reactions, various concentrations of the agents are added at the same time as labelled dCTP and the polymerase reactions are allowed to proceed. After an extraction of viral DNA and analysis by gel electrophoresis and autoradiography, the amount of labelled DNA from each reaction is quantified by densitometery of the audioradiogram. Densitometry data is fitted to logistic dose response curves using Tablecurve2D software.

EXAMPLE 17

1. Antiviral testing of the precore mutant (G1869A) HBV baculovirus

In this study, comparable HBV DNA production by wild-type (1.3 x genomic length HBV) and the precore mutant HBV was found using the recombinant HBV-baculovirus system. The dose effect of lamivudine, adefovir, and penciclovir on the wild-type HBV /bacuolvirus virus and the precore mutant (G1896A) HBV are shown in Figure 11 (A, B, C) and the calculated IC50 is shown in Table 3. HBV with the specific mutation of G1896A seemed to be at least as sensitive for adefovir, and may be more sensitive to lamivudine and penciclovir compared with wild type HBV.

2. Antiviral testing with the precore mutant (G1869A), anD L526M/M550V or M550I HBV baculovirus

Intracellular HBV DNA and extracellular virus produced by HepG2 cells transduced with various recombinant HBV/baculovirus (including L526M/M550V, precore/L526M/L550V, M550I and precore/M550I) are shown in Figure 12. The HBV DNA yield from the various mutants showed that wild-type and precore mutant were comparable, the M550I and L526M/M550V mutants showed much lower HBV DNA production than wild-type, and the precore/M550I and precore/L526M/552V mutants had similar levels of DNA production (both intracellular and extracellular) as the precore mutant. The presence of M550I and L526M/M550V changes did not seem to diminish their replication fitness compared to the precore mutant.

Intracellular HBV DNA and extracellular virus produced by HepG2 cells transduced with various recombinant HBV-baculovirus (including M550I, precore/M550I, L526M/M550V and precore/L526M/L550V) in the presence of adefovir, or lamivudine or penciclovir are shown in Figure 13 (A-F) and the calculated IC50 for adevoir is shown in Table 4. The adefovir concentration required to inhibit HBV replication (intracellular single-stranded DNA) by 50% (IC_{50}) was 0.94 μM and 0.93 μM for the recombinant HBV-baculovirus

mutants M550I and L526M/M550V respectively, and 0.28 μM and 0.47 μM for precore/M550I and precore/L526M/M550V respectively. The Southern blots of intracellular HBV replicative intermediates and extracellular virus produced by HepG2 cells transduced with respective recombinant HBV-baculovirus showed that for any HBV/baculovirus variant encoding the mutations at M550I or L526M/M550V changes conferred a high degree of resistance to lamivudine and penciclovir and no dose response could be plotted.

TABLE 3 *IC_{50} of adefovir, lamivudine and penciclovir for wild-type (WT) or precore (G1896A) recombinant HBV-baculovirus*

Drugs		WT	Precore
adefovir IC_{50} (μM)	IC SS[i] EC RC[j]	0.235[a] 0.0535[c]	0.195[b] 0.025[d]
lamivudine IC_{50} (μM)	IC SS EC RC	0.0697[e] 0.018[g]	0.0207[f] 0.011[h]
penciclovir IC_{50} (μM)	IC SS EC RC	226 23	85 6

[a] average of 2 experiments (0.25 and 0.22 μM)

[b] average of 2 experiments (0.20 and 0.19 μM)

[c] average of 2 experiments (0.06 and 0.047 μM)

[d] average of 2 experiments (0.02 and 0.03 μM)

[e] average of 3 experiments (0.067, 0.085 and 0.0572 μM)

[f] average of 3 experiments (0.014, 0.014 and 0.032 μM)

[g] average of 3 experiments (0.022, 0.0293 and 0.0095 μM)

[h] average of 3 experiments (0.012, 0.01 and 0.01 μM)

[i] intracellular single-stranded HBV DNA

[j] extracellular relaxed circular HBV DNA

TABLE 4 *IC_{50} of adefovir, lamivudine and penciclovir for recombinant HBV-baculovirus encoding changes conferring lamivudine resistance with or without the precore (G1896A) mutation*

Recombinant HBV-baculovirus		adefovir IC_{50} (µM)	lamivudine IC_{50} (µM)	penciclovir IC_{50} (µM)
M550I	IC SS	0.94	NDR	NDR
	EC RC	*	*	*
L526M/M550V	IC SS	0.93	NDR	NDR
	EC RC	*	*	*
Precore/M550I	IC SS	0.28	NDR	NDR
	EC RC	0.09	NDR	NDR
Precore/L526M/M550V	IC SS	0.47	NDR	NDR
	EC RC	1.0	NDR	NDR

NDR no dose response

* HBV DNA production to low to be measured.

IC SS intracellular single-stranded HBV DNA

EC RC extracellular relaxed circular HBV DNA

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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